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Engineered PQQ Glucose Dehydrogenase-Based Enzyme Sensor for Continuous Glucose Monitoring

Junko Okuda, Junko Wakai, Satoshi Igarashi, and Koji Sode*

Department of Biotechnology, Faculty of Technology, Tokyo University of Agriculture and Technology, Tokyo, Japan

ABSTRACT

Continuous glucose monitoring (CGM) is expected to become an ideal way to monitor the glycemic level of diabetic patients. A recent trend in the disposable self blood glucose sensing development has been the use of pyrroloquinoline quinone-harboring glucose dehydrogenase (PQQGDH). However, due to a number of limitations of PQQGDH, conventionally utilized glucose oxidase (GOD) remains widely utilized in CGM. Two major problems that arose in the application of PQQGDH for CGM are the poor stability and its requirement for artificial electron acceptors for electrochemical measurement. To solve these problems, we investigated the amenability of our engineered PQQGDH Ser415Cys, which has a far superior thermal stability over the wild-type enzyme, for

*Correspondence: Koji Sode, Department of Biotechnology, Faculty of Technology, Tokyo University of Agriculture and Technology, 2-24-16 Nakamachi, Koganei, Tokyo 184-8588, Japan; Fax: 81-42-388-7027; E-mail: sode@cc.tuat.ac.jp.

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the CGM system, and the applicability of cyt b_{562} as the electron mediator to construct a CGM system free of synthetic mediator. As a result, the operational stability of CGM system employing Ser415Cys co-immobilized with cyt b_{562} was far superior to that of the wild-type enzyme-based electrode, with more than 60% of the initial response observed after 72 hr at 37°C. We achieved the successful application of PQQGDH in continuous operation without a significant decrease in the sensor signal.

Key Words: Continuous glucose monitoring (CGM); PQQ glucose dehydrogenase (PQQGDH); Glucose sensor; Cytochrome b_{562} (cyt b_{562}).

INTRODUCTION

Continuous glucose monitoring (CGM) is expected to become an ideal way to monitor the glycemic level of diabetic patients, as well as an useful tool to control blood glucose level in combination with an insulin pump to realize the concept of artificial pancreas. CGM systems are basically composed of a continuous glucose enzyme sensor and sampling device, which either takes blood samples or interstitial fluid samples.[1–3] The core technology for the measurement is, therefore, the glucose enzyme sensor, which can be applied for continuous sensing. A recent trend in the disposable self blood glucose sensing (finger stick type) development has been the use of pyrroloquinoline quinone-harboring glucose dehydrogenase (PQQGDH), due to its insensitivity to oxygen, instead of the conventionally utilized glucose oxidase (GOD). However, due to a number of limitations of PQQGDH, GOD remains widely utilized in CGM, with H_2O_2-based monitoring utilizing oxygen as the electron acceptor. Two major problems that arose in the application of PQQGDH for CGM is the poor stability of the enzyme for continuous operation and its requirement for artificial electron acceptors for electrochemical measurement. The conventionally utilized electron mediators are low molecular weight, toxic metal-complexed compounds, thus preventing the use of PQQGDH for *in vivo* applications.

The authors have been carrying out protein engineering of PQQGDH as an attempt to overcome its inherent inferior properties vs. GOD for CGM applications.[4–6] One achievement has been the recently reported Ser415Cys mutant of PQQGDH whose homodimer structure is covalently bound by the introduction of a disulfide bond in its dimer interface.[7] The far superior thermal stability of Ser415Cys over the wild-type enzyme, without alterations to its catalytic activity, may overcome the stability

problem. To solve the mediator problem, we have also proposed the idea of utilizing cytochromes as an alternative to the artificial synthetic electron mediators for PQQGDH-based glucose sensors. We have focused on the application of *Escherichia coli* cytochrome b_{562} (cyt b_{562}), a 12.7-kDa monomeric periplasmic protein. We constructed enzyme electrodes co-immobilizing PQQGDH with cyt b_{562}, thus allowing glucose measurement without the use of an artificial electron mediator.[18,9]

In this paper, we first confirmed the amenability of engineered Ser415Cys PQQGDH for the CGM system, utilizing ferrocene as the electron mediator. We then investigated the applicability of cyt b_{562} as the electron mediator to construct a CGM system free of synthetic mediator.

MATERIALS AND METHOD

Materials

Recombinant PQQGDH (water-soluble PQQGDH, PQQGDH-B, or s-GDH) from *Acinetobacter calcoaceticus* and cyt b_{562} from *E.coli* were prepared using *E.coli* DH5α as host strain as previously described.[15–8]

Enzyme Sensor Construction and Operation

PQQGDH/ferrocene-immobilized electrodes were made by mixing 20 mg of carbon paste (0.5 g graphite powder mixed with 0.3 mL paraffin liquid BSA Inc., West Lafayette, USA) with 100 U of wild-type PQQGDH (1.4×10^{-10} mol) or Ser415Cys (2.8×10^{-10} mol) in 10 mM MOPS buffer, pH 7.0. Bovine serum albumin (BSA) was added to each mixture to ensure that they contained an equal amount of protein. The mixtures were lyophilized and mixed with 10 mg of ferrocene. The mixtures were packed into the ends of carbon paste electrodes (dual type of carbon paste electrode for the flow cell from BAS Inc., Model No.11-1004 inner diameter is 3 mm, and geometric surface area of one hole is 0.28 cm^2), fixed with 1% glutaraldehyde solution for 30 min, and washed with 10 mM Tris–HCl buffer (pH 7.0). Recombinant PQQGDH was prepared in the apo-form, without PQQ in its active center. To allow hole formation, the electrodes were soaked in 10 mM MOPS buffer (pH 7.0) containing 5 μM PQQ and 1 mM $CaCl_2$ at 4°C for at least 30 min, washed with 10 mM MOPS buffer (pH 7.0), and stored at 4°C until use.

Electrodes with co-immobilized PQQGDH/cyt b_{562} were prepared as described above, except that ferrocene and BSA were omitted. Instead, a 100 molar excess of cyt b_{562} was mixed with 20 mg of carbon paste and 100 U

of wild-type PQQGDH or Ser415Cys. The lyophilized mixtures were packed and fixed into electrodes, and holo formation was allowed to proceed as described above.

The enzyme electrode, reference electrode, and counter electrode were joined to a cross-flow cell (BAS Inc. Model No.11-2456) that was connected to a flow pump (CCPM, TOHSOH) in a column oven (Co8020, TOHSOH, Japan), as depicted in Fig. 1. An Ag/AgCl electrode (Model RE-3V, BAS Inc.) and a stainless steel tube (BAS Inc) were used as reference and counter electrodes, respectively. The carrier solution was 10 mM MOPS buffer (pH 7.0) containing 1 mM $CaCl_2$ with flow rate of 0.5 mL min^{-1}. The potential was controlled by a potentiostat HA151 (HOKUTO-DENKO, Tokyo, Japan) in a three-electrode cell and currents were recorded with a chart recorder (Ohkura electric company, Tokyo, Japan). The applied potential for the measurements was +200 or +250 mV vs. Ag/AgCl for PQQGDH/ferrocene or PQQGDH/cyt b_{562} electrode. CGM carried out by measuring the current response to 5 mM glucose with flow rate of 0.1 mL min^{-1} for 72 hr. All measurements were performed at 37°C.

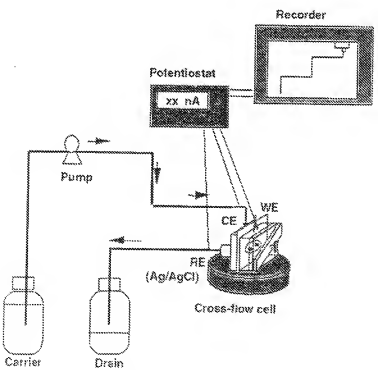

Figure 1. Schematic diagram of CGM system.

RESULTS AND DISCUSSION

Investigation of Operational Stability of CGM System Employing PQQGDH

We first investigated the operational stability of wild-type PQQGDH and the engineered PQQGDH, Ser415Cys, in the CGM system utilizing the synthetic electron mediator ferrocene. The wild-type PQQGDH electrode, with ferrocene as the electron mediator, responded immediately after supplying carrier solution containing glucose and reached steady state within 5 min (Fig. 2). Based on the observed steady state currents resulting from increasing glucose concentrations, calibration curves were constructed for the wild-type PQQGDH- and Ser415Cys-based electrodes, respectively (Fig. 3). Good linear correlations were observed in both systems between steady state current and glucose concentration below 5 mM.

The operational stability of the CGM system was then investigated for both electrodes by a time course of continuous operation at 37°C with 5 mM glucose, corresponding to the approximate glucose concentration in healthy humans (Fig. 4). After 72 hr of operation, the wild-type GDH electrode maintained only 25% of the initial response. The calibration curve determined after 72 hr of operation [Fig. 3(a)] shows a general decrease in current response to about 30% of the original value. These results indicate that wild-type PQQGDH is readily and irreversibly inactivated during the continuous operation for 72 hr.

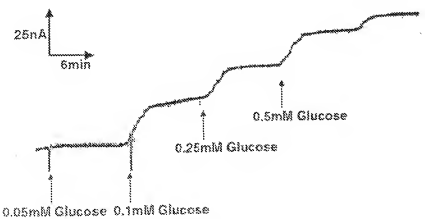

Figure 2. Response curve of the sensor employing wild-type PQQGDH and ferrocene as mediator.

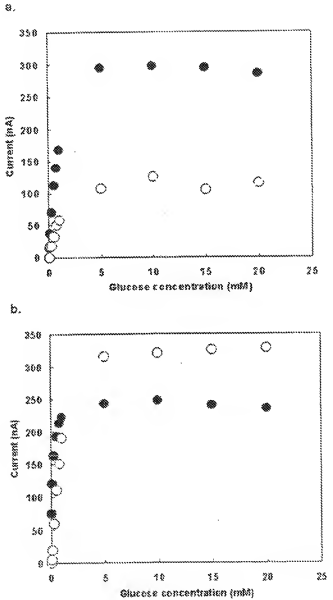

Figure 3. Calibration curves of PQQGDH/ferrocene electrodes before operation (●) and after 72 hr operation (○). (a) Wild-type PQQGDH/ferrocene electrode. (b) Ser415Cys/ferrocene electrode. The carrier solution was 10 mM MOPS buffer (pH 7.0) containing 1 mM $CaCl_2$ with flow rate 0.5 mL·min^{-1}. The applied potential for the measurements was +200 mV vs. Ag/AgCl. CGM was carried out by measuring the current response to 5 mM glucose with flow rate 0.5 mL·min^{-1} for 72 hr. All experiments were done at 37°C.

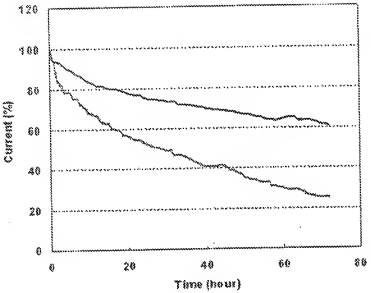

Figure 4. Operational stability of PQQGDH/ferrocene electrodes. Wild-type PQQGDH (·····) or Ser415Cys (——) was immobilized with ferrocene. The carrier solution was 10 mM MOPS buffer (pH 7.0) containing 1 mM $CaCl_2$ and 5 mM glucose with flow rate 0.1 mL·min^{-1}. The applied potential for the measurements was +200 mV vs. Ag/AgCl. CGM was carried out by measuring the current response to 5 mM glucose with flow rate 0.5 mL·min^{-1} for 72 hr.

In contrast, the CGM system employing the thermostable engineered Ser415Cys PQQGDH maintains 60% of the initial response after 72 hr of continuous operation (Fig. 4). The half-life of Ser415Cys-based CGM system was calculated at 152 hr, which reflects a far greater stability than the CGM system employing the wild-type enzyme (half-life 52.4 hr). The calibration curve of the Ser415Cys electrode after 72 hr of operation showed responses that were only slightly lower than initial values [Fig. 3(b)]. Therefore, the observed inactivation of the engineered enzyme was reversible and was recovered by incubation in glucose free carrier solution, which was carried out between these experiments, although wild-type enzyme activity was not recovered.

The quaternary structure of Ser415Cys is greatly stabilized by a disulfide bond introduced in its dimer interface.[7] These results demonstrate that Ser415Cys is a stable enzyme to be utilized in CGM system. The inactivation of this engineered PQQGDH during the continuous operation therefore likely results from the accumulation of enzyme reaction products as well as the reduced enzyme form.

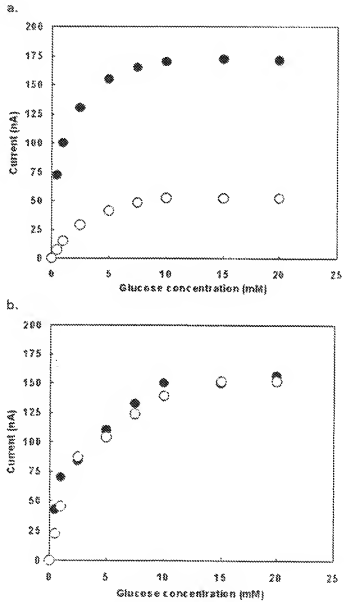

Figure 5. Calibration curves of PQQGDH/cyt b_{562} electrodes before operation (●) and after 72 hr operation (○). (a) Wild-type PQQGDH/cyt b_{562} electrode. (b) Ser415Cys/cyt b_{562} electrode. The carrier solution was 10 mM MOPS buffer (pH 7.0) containing 1 mM $CaCl_2$ with flow rate 0.5 mL min^{-1}. The applied potential for the measurements was +250 mV vs. Ag/AgCl. CGM was carried out by measuring the current response to 5 mM glucose with flow rate 0.5 mL min^{-1} for 72 hr. All experiments were done at 37°C.

CGM System Employing PQQGDH and Cyt b_{562}

We previously reported that cyt b_{562} can be utilized as the electron acceptor for PQQGDH.[8,9] On the basis of this observation, we investigated CGM system employing cyt b_{562} as the electron mediator co-immobilized with either wild-type PQQGDH or Ser415Cys PQQGDH. The glucose calibration curves of both CGM systems showed that cyt b_{562} can be utilized as the electron mediator in PQQGDH-based CGM systems (Fig. 5). The sensor responses were not so low compared with those for CGM systems employing the ferrocene mediator.

The time course of continuous operation for CGM systems employing PQQGDH/cyt b_{562} co-immobilized electrodes (Fig. 6) was almost identical to that obtained with the ferrocene mediator. The response of the wild-type PQQGDH-based CGM system decreased rapidly, with a half-life of

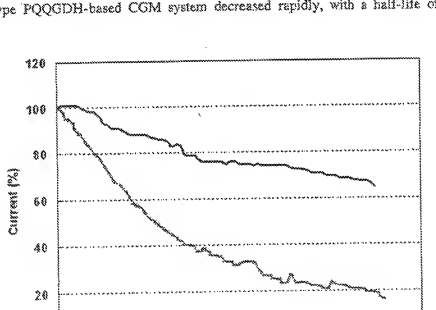

Figure 6. Operational stability of GDH/cyt b_{562} electrode. Wild-type PQQGDH (·····) or Ser415Cys (——) was co-immobilized with cyt b_{562}. The carrier solution was 10 mM MOPS buffer (pH 7.0) containing 1 mM $CaCl_2$ and 5 mM glucose with flow rate 0.1 mL min^{-1}. The applied potential for the measurements was +250 mV vs. Ag/AgCl. CGM was carried out by measuring the current response to 5 mM glucose with flow rate 0.5 mL min^{-1} for 72 hr. All experiments were done at 37°C.

approximately 37 hr. After 72 hr of operation less than 20% of initial response was observed. The calibration curve determined after 72 hr of continuous operation [Fig. 5(a)] revealed that wild-type enzyme was inactivated, with over 70% decreased signals.

The operational stability of CGM system employing Ser415Cys co-immobilized with cyt b_{562} was far superior to that of the wild-type enzyme-based electrode, with more than 60% of the initial response observed after 72 hr (half-life 144 hr). The calibration curve investigated after 72 hr of operation was indistinguishable from that obtained before the operation [Fig. 5(b)]. These results confirm that cyt b_{562}, together with the engineered PQQGDH, has sufficient stability for continuous operation at 37°C.

We achieved for the first time, the successful application of PQQGDH in continuous operation without a significant decrease in the sensor signal. Moreover, this is also the first report of the construction of a CGM system free of synthetic electron mediator. Further optimization of the sensor system is expected to avoid the decrease in signal during operation, since the engineered enzyme was not irreversibly inactivated and the natural cyt b_{562} electron mediator was also stable. Future evaluation of the cytotoxicity of cyt b_{562} may result in the achievement of an implantable-type CGM system employing cyt b_{562} as the electron mediator.

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